REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

I. Status of the Claims

With this submission, claim 1 is amended and claim 18 is newly added. Support for these amendments can be found throughout the specification, and specifically on page 20 lines 20-24. No claims are cancelled. Hence, upon entry of this paper, claims 1-4 and 6-18 will remain pending with claims 1, 2, 6-9, 13 and 17-18 under active consideration.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

II. Rejections Withdrawn

Applicants wish to thank the Examiner for withdrawing the objections to the claims.

III. Claim Rejection - 35 U.S.C. §103(a)

Claims 1-2, 6-7, 13 and 17 are rejected as allegedly obvious over Harlow & Lane ("Harlow") in view of Ishikawa et al. (Genomics 26(3):527-34)("Ishikawa"), U.S. 5,623,053 ("Gastinel"), U.S. 5,639,597 ("Lauffer"), U.S. 5,866,693 ("Laping") and U.S. 5,541,087 ("Lo"). The Office admits that Harlow "fail to teach soluble HM1.24 antigen protein as the type of antigen, and similarly fail to teach anti-HM1.24 antibodies as the type of antibodies detected." (Office Action, page 5) Nevertheless, the Office combines Harlow with Ishikawa, which teaches use of the soluble HM1.24 antigen protein lacking 18 amino acid residues from the C-terminal of SEQ ID NO:20. However, the Office recognizes that the claimed invention recites a protein modified by lacking 17 amino acid residues from the C-terminus of SEQ ID NO:20. To remedy this deficiency, the Office states that "the resulting protein lacking 17 rather than 18 amino acids would be reasonably expected to have the same properties." (Office Action, page 13) Applicants respectfully traverse the rejection.

The present invention relates to measurement of a concentration of an anti-HM1.24 antibody present in a test sample. In Example 10, serum of Rhesus monkey was used. As well known in the art, serum contains various proteases, and therefore when serum and a protein (for the present case, a soluble HM1.24 antigen protein) are mixed for an ELISA assay, the protein is hydrolyzed by the proteases in the blood, resulting in the decrease of stability and shortening of half-life of the protein. In other words, as far as a serum sample used in an ELISA assay is concerned, the protein has the same properties as the pharmacokinetic properties shown by proteins in the blood. This situation is the same as that when the protein is fluids (body fluids).

The IgG1 used in the Ishikawa reference was obtained from Dr. Seed. (See section "Production of soluble recombinant BST-2-immunoglobulin fusion protein" on page 528, right column.) Applicants enclose the Seed et al. reference that describes the fusion protein (DNA Cell Biol. 9(5): 347-353 (1990), "Seed") (Exhibit A). Specifically, the Seed reference describes that the serum survival (such as half-life) is **prolonged** by fusion to IgG1-Fc. Therefore, it is clear that Ishikawa used the IgG1 Fc for improving the serum survival (such as half-life). Therefore, it cannot be considered that a person of ordinary skill would delete the IgG1 Fc so as to worsen the serum survival.

According to the present invention, it was newly found that a soluble HM1.24 antigen protein lacking the C-terminal 17 amino acids can form a dimer without the need for addition of an Fc sequence for dimer formation. Additionally, the soluble HM1.24 antigen of the present invention maintains the biological activities of the HM1.24 antigen protein, which is necessary for the purpose of the present invention (see page 7, lines 21-26 and page 59, lines 10-14 of the specification).

Furthermore, a proposed modification to the prior art cannot render the prior art unsatisfactory for its intended purpose. If a proposed modification would render the prior art invention unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 211 USPQ 1125 (Fed. Cir. 1984). MPEP 2143.01 Section V. Additionally, MPEP § 2143.03(VI) states that "[a] prior art reference must be considered in its entirety, i.e., as a whole, including portions that would

lead away from the claimed invention." Accordingly, where cited art teaches away from a claimed feature, the cited art is not available for the purposes of an obviousness rejection.

Thus, one of skill in the art would not be motivated to remove the Fc sequence for use to detect or determine a soluble HM1.24 antigen protein at low concentrations. As provided in the prior art, the purpose of fusing Bst-2 to an Fc sequence is to form a dimer at the Fc region so as to improve and enhance the pharmacokinetic properties (especially stability, half-life, activity, etc.) of Bst-2. Removal of the Fc region would counter what a person of ordinary skill in the art would be trying to achieve - a more stable Bst-2 for detection or determination of a soluble HM1.24 antigen protein at low concentrations.

Therefore, for at least these reasons, Applicants respectfully request reconsideration and withdrawal of the rejection.

IV. Claim Rejection - 35 U.S.C. §103(a)

Claims 8-9 are rejected as allegedly obvious over Harlow in view of Ishikawa, Gastinel, Lauffer, Laping, Lo and further in view of U.S. 5,646,115 ("Frank"). The Office admitted that Harlow, Ishikawa, Gastinel, Lauffer, Laping, and Lo "fail to specifically teach using a second antibody in addition to the antibody specific to test the antibody" (Office Action, page 16). Nevertheless, the Office pointed to Frank as teaching "the amount of antibody bound to the solid phase can be determined using one or more layers of secondary antibodies." Office Action, page 16.

Frank does not cure the deficiencies of the other cited references. Specifically, Frank does not teach or even suggest that the Fc region should be removed from the antigen.

Thus, for at least these reasons, Applicants respectfully request the rejection be withdrawn.

CONCLUSION

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing or a credit card payment form being unsigned, providing incorrect information resulting in a rejected credit card transaction, or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

By _____

Stephen B. Maebius
Attorney for Applicant
Registration No. 35,264

Date August 13, 2010

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5569
Facsimile: (202) 672-5399

EXHIBIT A

DNA AND CELL BIOLOGY
Volume 9, Number 5, 1990
Mary Ann Liebert, Inc., Publishers
Pp. 347-353

Expression and Characterization of Human CD4: Immunoglobulin Fusion Proteins

GERD ZETTLMEISSL, JENS-PETER GREGERSEN, JEAN MICHEL DUPORT, SABINE MEHDI, GÖTZ REINER, and BRIAN SEED*

ABSTRACT

Different chimeric antibody-like molecules consisting of the four human CD4 extracellular domains (amino acids 1–369) fused to different parts of human IgG_1 and IgM heavy-chain constant regions have been created and expressed in mammalian cells. For both IgG_1 and IgM fusion proteins, the best expression in COS cells was observed for molecules lacking the CH1 domain of the heavy-chain constant region. The chimeric molecules are potent inhibitors of human immunodeficiency virus (HIV) infection and HIV-mediated cytotoxicity. A CD4:IgG_1 hinge fusion protein, which was analyzed in more detail, binds efficiently to HIV gp160 and human Fc receptors and shows complement-assisted inhibition of viral propagation in culture. Half-life studies after intravenous application of the latter human fusion protein into mice and monkeys showed significant prolongation of serum survival compared to soluble CD4. An IgG_{2b} murine homolog of the human CD4:IgG_1 hinge fusion protein was prepared and evaluated in mice, where it was found to be nontoxic and to have no detectable effect on the humoral response to soluble antigen.

INTRODUCTION

Most patients infected with the human immunodeficiency virus (HIV) develop acquired immunodeficiency syndrome (AIDS) (Curran *et al.*, 1988), which is characterized by the progressive depletion of T cells expressing CD4, the cellular receptor for HIV (Sattenau and Weiss, 1988). Soluble CD4 molecules have been shown to interfere with HIV-1 infection and HIV-mediated syncytium formation *in vitro* (Smith *et al.*, 1987; Deen *et al.*, 1988; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988; Clapham *et al.*, 1989). Recent trials in monkeys have shown that soluble CD4, if administered to infected animals without marked CD4 cell cytopenia, can reduce simian immunodeficiency virus (SIV) titers and improve *in vitro* measures of myelopoiesis (Watanabe *et al.*, 1989). However the SIV titer was found to rise and myelopoietic potential to decline after treatment was discontinued, suggesting that continuous lifetime treatment might be necessary to stave off progressive encroachment of the immune system.

In vitro, CD4 immunotoxin conjugates or fusion proteins have been shown to actively target infected cells for killing (Chaudhary *et al.*, 1988; Till *et al.*, 1988). Unfortunately, because toxins are foreign proteins, they are likely susceptible to immune recognition and clearance if administered repeatedly in a clinical setting.

The ideal immunotoxin would combine natural immune effector function with a specific recognition element directed against the pathogen of interest. If the recognition element is the receptor by which the pathogen gains entry to its host cells, the pathogen cannot mutate away from the immunotoxin and still retain its virulence. Recently, Capon *et al.* (1989) published the expression and properties of CD4:human IgG_1 fusion proteins bearing the CH1 domain, whereas Traunecker *et al.* (1989) showed the expression and characterization of CD4:mouse IgG_{2a} and CD4:mouse IgM molecules lacking the CH1 domain.

In this report we investigate the expression and secretion in mammalian cells and the quaternary structure of CD4:human IgG_1 and CD4:human IgM chimeras harboring the extracellular domain of CD4 and different amounts of the immunoglobulin heavy-chain constant region. We present evidence that chimeras consisting of the CD4 extracellular

Research Laboratories of Behringwerke AG, D-3550 Marburg, West Germany.
*Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114.

domain fused to the various human antibody effector regions are potent inhibitors of HIV infectivity. The properties of the most efficiently secreted CD4:IgG_1 hinge fusion protein were analyzed in more detail. This molecule binds to HIV gp160 and to different human Fc receptors and exhibits long serum half-lives in mice and monkeys. In long-term assays it shows complement-assisted restriction of viral propagation. Administration of the molecule and its mouse L3T4:IgG_{2b} homolog to mice does not compromise their ability to respond to soluble antigen.

MATERIALS AND METHODS

Purification of fusion proteins from COS cells

IgG_1 fusion proteins were adsorbed in batches to Protein A Tris-acryl (Pierce), harvested by filtration, washed in a column with at least 20 volumes of PBS containing 1% Nonidet P-40, with 5 or more volumes of 0.15 *M* NaCl, 1 m*M* EDTA pH 8, and were eluted with 0.1 *M* acetic acid. IgM fusion proteins were similarly harvested and washed after adsorption to agarose beads coupled (by CNBr activation) with 1 mg/ml of rabbit anti-human IgG affinity-purified antibody (Calbiochem). The anti-IgM agarose columns were eluted with 0.2 *M* glycine-HCl buffer pH 2.5. Eluted fusion proteins were neutralized with 2.0 *M* Tris base and concentrated in centrifugal ultrafilters (Amicon).

Stable BHK cell line transfectants

BHK cells in DME/10% fetal bovine serum (FBS) were transfected with the CD4:IgG_1 hinge fusion gene (20 μg), pSV2dhfr (5 μg), and pRMH140 (neo^r, 5 μg) as described (Zettlmeissl *et al.*, 1988). Three days later, cells were split 1:3 to 1:4 into DME/10% FBS containing 400 μg/ml G-418 and 1 μM methotrexate. Individual colonies were picked and screened for relative expression, and a single isolate (BHK-UC3) was expanded for further analysis. To purify fusion proteins, conditioned culture supernatants of BHK-UC3 cells grown in protein-free medium were filtered, adjusted to 19 m*M* Tris HCl pH 8.6, and adsorbed to a Protein A-Sepharose column. The column was washed with 10 volumes of 150 m*M* NaCl, 50 m*M* Tris-HCl pH 8.6, and eluted with 0.1 *M* sodium citrate pH 3. The eluate was adjusted to pH 8 with Tris base and dialyzed against 50 m*M* Tris-HCl pH 7.4 containing 50 m*M* NaCl and 1 m*M* EDTA (TNE-buffer). The resulting protein was >95% pure by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Syncytium assay

HPB-ALL cells were infected with the VCS-25 vaccinia recombinant bearing the HIV-1 envelope-coding sequence. Twelve hours post infection, the cells were divided into microtiter wells and purified, or partially purified fusion proteins were added to each well. OKT4a monoclonal antibody was added as a positive control, and mouse L3T4:IgG_{2b} hinge fusion protein or human CD2:IgG_1 hinge fusion protein was added as a negative control. After 8 more hr of incubation, the cells were washed with PBS, fixed with PBS containing 4% paraformaldehyde, and microscopically analyzed for the presence of giant cells. Cultures without giant cells were considered as negative.

gp160 binding assay

Purified recombinant gp160 from vaccinia virus-infected mammalian cells (kindly provided by Transgene and Pasteur Vaccin) was radioiodinated with lactoperoxidase to a specific activity of 4.5 μCi/μg. Sixty nCi of labeled protein were diluted with increasing amounts of unlabeled gp160 and incubated with CD4:IgG_1 hinge fusion protein (2.7 n*M*) in TNE-buffer in a final volume of 0.2 ml. After 1 hr of incubation at 4°C Protein A-Sepharose (50 μl of a 10% suspension in phosphate-buffered saline containing 1% Triton X-100 and 1 m*M* EDTA; PBSTE) was added and allowed to incubate 2 hr further; bound and free fractions were separated by centrifugation of the beads and washing with 200 μl of PBSTE and PBSTE containing 0.2% $NaDoDSO_4$. The data were corrected for nonspecific binding, determined as counts bound in the absence of fusion protein.

RESULTS

CD4:immunoglobulin fusion genes were created by fusing a CD4 cDNA to human IgG_1 and IgM genomic sequences. The fusions were accomplished with the aid of a joining oligonucleotide encoding a 5-amino-acid linker sequence (HADPE) and a synthetic splice donor sequence, which allowed the reading frame to be preserved between CD4 and the various Ig exons to which it was fused (Fig. 1). Five human fusion genes were created in which the portion encoding the extracellular domain of CD4 (amino acids 1–369) and the 5-amino-acid linker sequence were placed upstream from the CH1, hinge, or CH2 exons of the human IgG_1 gene, or upstream from the CH1 or CH2 exons of the IgM gene. Four similar constructs were prepared from the mouse L3T4 gene and murine IgG_{2b} or IgM chromosomal sequences; however, an L3T4 fusion to CH2 was not created. Transient expression of the fusion proteins in COS cells resulted in the secretion of monomeric (CH2 fusion) or dimeric (CH1 and hinge fusion) immunoglobulin-like molecules in the human IgG_1 case, and large multimeric molecules beyond the resolution of the gel system used in the human IgM case (Fig. 2A). In general, poor expression was observed for fusion proteins bearing CH1 domains from either murine or human immunoglobulins (Fig. 2 and data not shown).

To prepare large amounts of a highly expressed fusion protein, a BHK cell stable transfectant was prepared from the CD4:IgG_1 hinge fusion construct. From the transfectant supernatant a final purified yield of 5–15 μg/ml/day was obtained. Gel electrophoretic analysis of the purified protein showed dimeric molecules, with an apparent subunit molecular mass of 75 kD (Fig. 2B). Equilibrium analytical ultracentrifugation showed a single molecular species

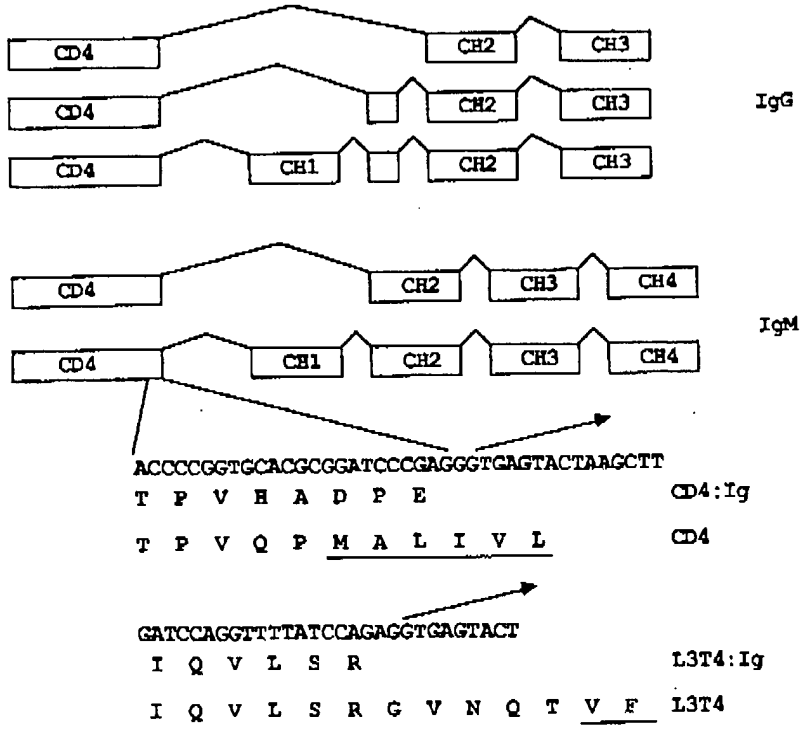

FIG. 1. Structure of the human and murine Ig fusion genes. Human (CD4) and murine (L3T4) synthetic splice donor sequences and their translation products are shown below a schematic diagram of the fusion genes. The native transmembrane domain is underlined.

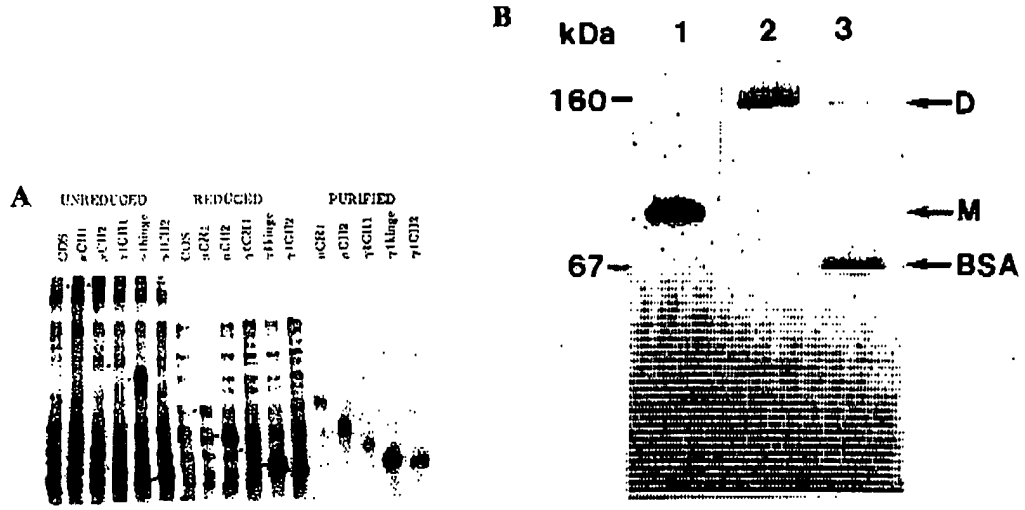

FIG. 2. Expression of the human fusion genes in COS cells (a) and BHK cells (b). a. Cells were transfected with the indicated CD4:Ig fusion genes cloned in a CDM8-based expression vector (Seed, 1987) as previously described (Seed and Aruffo, 1987). At 40 hr post transfection, the medium was aspirated and replaced by serum-free DME medium lacking cysteine and methionine (GIBCO), containing 200 μCi/ml of [^{35}S]methionine and $\approx$40 μCi/ml of [^{35}S]cysteine (Translabel, ICN). Eight hours later, the medium was removed and aliquots, either reduced or nonreduced, were applied to a 5% polyacrylamide gel. Fusion proteins were purified as described in the Materials and Methods. b. CD4:IgG$_1$ hinge fusion protein prepared from a stable BHK cell transfectant. Reduced (lane 1) and unreduced (lane 2) fusion protein prepared from BHK cells are displayed alongside to mouse IgG and BSA markers (lane 3).

with a mass of 145 ± 12 kD (R. Jaenicke, personal communication). Amino-terminal sequence analysis yielded KKVVLGKKGD (B. Siebold, personal communication) in accord with the known sequence.

Pure or partially purified proteins secreted from COS cells were assayed for inhibition of syncytia formed between HPB-ALL (T-cell leukemia) cells infected with a vaccinia virus recombinant (Chakrabarti *et al.*, 1986) bearing the HIV envelope coding region. Blockade of syncytium formation was observed at a concentration of 20 μg/ml for all but the IgG_1 CH1 fusion protein, whose limited availability allowed testing only at 5 μg/ml. At this concentration, partial blockade was observed. The CD4:IgG_1 hinge fusion protein purified from BHK cells was analyzed in more detail and also proved effective at blocking HIV-1 replication in a long-term virus neutralization assay over 5 weeks in Jurkat cells. Cultures with either reverse transcriptase activity (Gregersen *et al.*, 1988) or giant cell formation were considered as positive. The 50% neutralizing concentration for infection of cultures with 100 tissue culture infectious doses (TCID) was I μg/ml, and the inclusion of guinea pig complement provided complete neutralization at this concentration (data not shown). Moreover, the BHK-prepared CD4:IgG_1 hinge fusion protein efficiently suppressed the spread of virus from previously infected cells to uninfected cells cocultured with them for long periods of time (Table 1). When infected cultures were treated with CD4 fusion protein in the presence of complement for extended periods of time, a decrease in reverse transcriptase activity was seen over that measured in cultures treated with CD4 fusion protein alone (Table 1).

Despite this, neither the IgG nor the IgM fusion proteins displayed significant complement-dependent activity in a short-term chromium release assay with cells infected by a vaccinia recombinant expressing HIV envelope sequences (data not shown).

The ability of the purified CD4:IgG_1 hinge protein to bind to human macrophage high- and low-affinity Fc receptors was measured by displacement analysis of the binding of radiolabeled IgG_1 to COS cells transfected with cDNAs encoding FcRI, FcRIIa, or FcRIIb (Stengelin *et al.*, 1988; Allen and Seed, 1989). The CD4 fusion protein showed an affinity for the three receptors that was the same, or slightly higher, than the affinity for IgG_1 itself (Fig. 3A–C). In spite of this result, we observed no enhancement of infection of human peripheral blood macrophages (monitored by an antigen ELISA for HIV-1/p24) when infectious doses of HIV-1 (5–50 $TCID_{50}$) were preincubated with up to 50 μg/ml of the CD4:IgG_1 hinge protein (for experimental details, see Gregersen *et al.*, 1990).

Scatchard analysis of the binding of ^{125}I-labeled recombinant gp160 gave a dissociation constant of 7.6 ± 1.7 × 10^{-9} *M* (Fig. 3D), which relates very closely with published dissociation constants for soluble recombinant CD4 (Capon *et al.*, 1989).

Because antibodies are among the longest lived of circulation proteins, CD4:Ig fusion proteins might be expected to show increased serum survival. To test this, purified protein prepared from the BHK transfectant was injected intravenously into mice and cynomolgus monkeys (*Macacca fascicularis*). Persistence of the intact fusion protein was measured by a specific capture ELISA assay using solid-phase anti-CD4 antibody and an anti-immunoglobulin solution phase second antibody reagent. The plasma half-life of the protein determined from the asymptotic decay behavior was 12 hr in monkeys and 14 hr in mice (Fig. 4). These values are about 50-fold higher than the re-

TABLE 1. EFFECT OF CD4:IgG_1 HINGE FUSION PROTEIN ON HIV-1-INFECTED CELLS

Treatment on days 0, 8, and 11 with:	*CD4:IgG_1 (10 μg/ml)*		*CD4:IgG_1 (10 μg/ml) + complement*		*Complement*		*TNE buffer*	
Infection parameters:	*RT*[a]	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*	*RT*	*Fusions*
Day 0	ND[b]	+	ND	+	ND	+	ND	+
Day 3	2.8	–	1.5	~	2.1	+ +	3.8	+ + +
Day 7	2.0	+	1.8	+	4.3	+ +	12.9	+ + +
Day 10	2.7	+	1.5	+	16.4	+ +	49.9	+ + +
Day 14	2.2	+	1.0	+	26.8	+ +	31.3	+ + +
Day 17	1.8	+	1.0	–	25.6	+ +	20.7	+ +
Day 21	2.3	+	1.8	–	27.0	+ +	21.3	+ +
Day 24	2.5	+	0.7	–	13.3	+ +	10.5	+ +
Day 28	7.7	+	1.1	–	9.6	+	7.4	+

[a]RT, Reverse transcriptase activity.
[b]ND, Not determined.

Infected H9 cells were mixed 1:10 into cultures of uninfected H9 cells (10^7 cells in 10 ml). Duplicate cultures were treated with CD4:IgG_1 hinge fusion protein, guinea pig complement (1:30), a combination of both, and TNE-buffer as control at the indicated days. Fifty percent of the culture medium of all cultures was replaced twice weekly, and once a week up to 50% of the cells were removed to adjust cell densities to constant values between 1–2 × 10^6 cells/ml.

Reverse transcriptase activity was determined according to Gregersen *et al.* (1988) and is given as sample/control ratio using supernatants of uninfected cultures as control. Sample/control ratios above 2 are considered as positive.

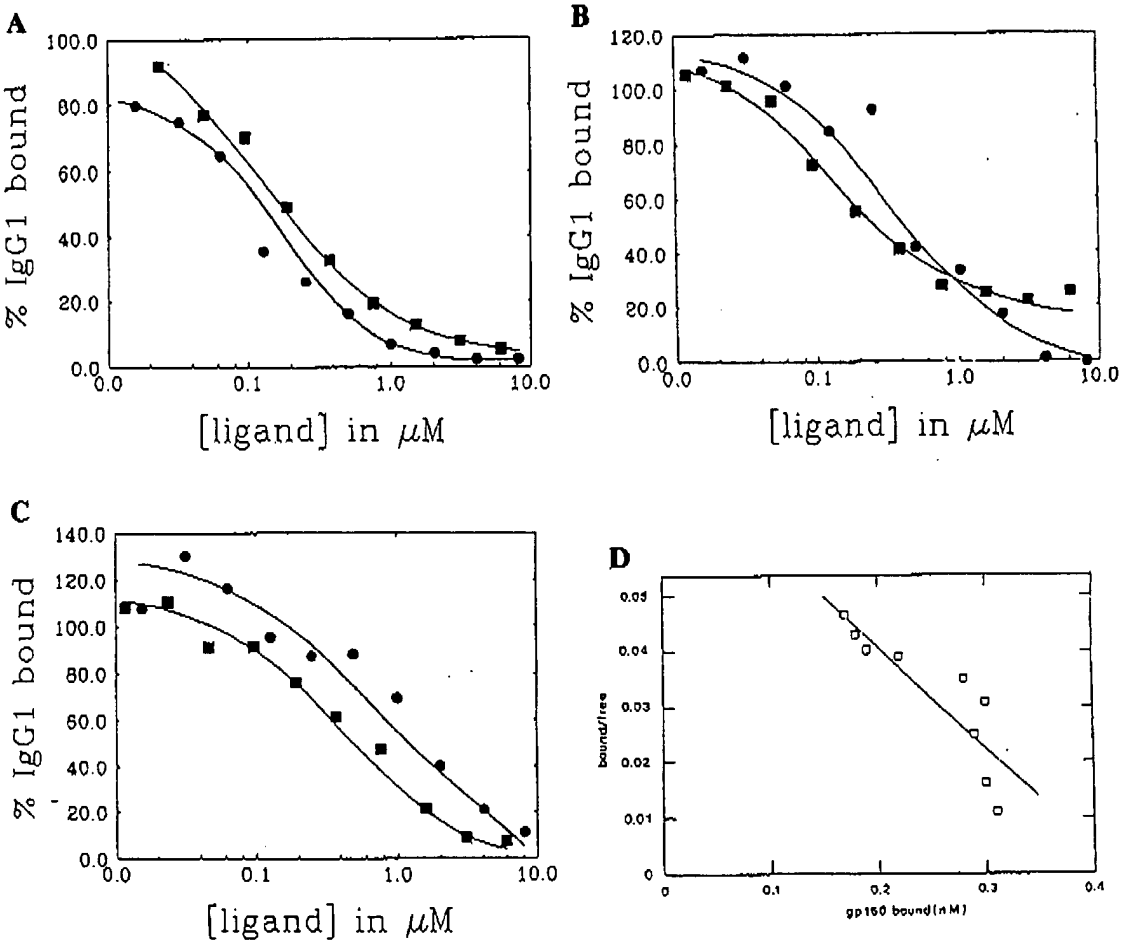

FIG. 3. CD4:IgG$_1$ hinge fusion protein binding analyses. a–c. Displacement analysis of binding of CD4:IgG$_1$ hinge fusion protein to Fc receptors expressed by COS cells. Displacement of ^{125}I-labeled human IgG, from COS cells transfected with high- (FcRI) (a) or low- (FcRIIa, FcRIIb) (b,c) affinity receptors was carried out as described (Stengelin *et al.*, 1988). (●) Displacement by fusion protein; (■) displacement by unlabeled IgG$_1$. d. Scatchard analysis of CD4 fusion protein binding to labeled HIV gp160. The solid line represents the best fit by least-square regression analysis to the mean values of five independent determinations.

ported value for the half-life of soluble CD4 in rabbits, but about 4-fold lower than reported for a fusion protein bearing the first two domains of CD4 fused to the human IgG$_1$ CH1 domain (Capon *et al.*, 1989).

Preliminary toxicological studies were carried out with human and murine fusion proteins in mice. On day 1, groups of 10 mice (5 male and 5 female) were injected intravenously with 80 μg per animal of either purified human CD4:IgG$_1$ hinge fusion protein, purified murine L3T4:IgG$_{2b}$ hinge fusion protein, or phosphate-buffered saline. The animals were observed for 30 days and were weighed on study days 0, 1, 7, and 14. No adverse reactions or change in body weight were observed. On day 2, 7, and 17, blood cell numbers including leukocytes, erythrocytes, platelets, hemoglobin, hematocrit, and mean cell volume were determined in 2 animals of each group; here too, no significant changes were observed. On day 14 the animals were reinjected as on day 1. On day 16 the animals were immunized with tetanus toxoid (TETANOL, Behringwerke AG, Marburg, FRG; 0.5 ml by intraperitoneal injection). Anti-toxoid antibody titers of sera collected on day 30 were determined by a specific ELISA. Control animals showed end point dilution values of 1:11,900 ± 5,500, while animals injected with the human and murine fusion proteins gave end point dilution values of 1:14,100 ± 6,400 and 1:9,200 ± 4,600, respectively.

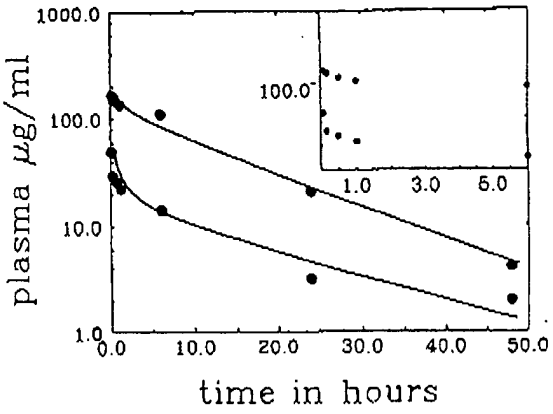

FIG. 4. Serum half-life of human CD4:IgG$_1$ hinge fusion protein in mice (lower) and monkeys (upper). The inset shows the first five samples of each experiment in expanded scale. For the mouse experiment, two groups of 3 animals were injected in the tail vein with ~100 μg of purified CD4:IgG$_1$ hinge fusion protein per mouse. From one group, blood samples were collected by retro-orbital bleeds 5 min, 30 min, 6 hr, and 48 hr after injection; from the other, samples were collected 10 min, 1 hr, and 24 hr after injection. For the monkey experiment, two cynomolgus monkeys with a body weight of 1.4 kg were injected in the saphenous vein with a bolus of 5 mg of purified fusion protein. One-milliliter blood samples were collected from the femoral vein at the same time intervals as for the mouse experiments. Serum concentrations of fusion protein were measured by a specific capture ELISA. Serum samples were incubated in microtiter wells coated with anti-CD4 antibody BMA041 (Behringwerke). The wells were washed, and developed with an alkaline phosphatase coupled antibody to human IgG (Zymed). The data for all animals are pooled in the figure shown.

DISCUSSION

Chimeric proteins bearing the extracellular domains of cell-surface proteins fused to immunoglobulin constant domains show promise as therapeutic agents. One of the most important issues confronting these agents is the extent of autoimmune damage arising from interaction of the fusion protein with its native ligand. Fortunately, the affinity of CD4 for its presumptive natural ligand, HLA class II antigen, is very low, and the affinity for HIV envelope is quite high. We find here, using a mouse model, that the fusion molecules have low toxicity in animals, and do not interfere with the humoral response to soluble antigen *in vivo*, consistent with the expectations raised by *in vitro* studies.

High-level expression of immunoglobulin fusion proteins was obtained following either transient or stable expression in nonlymphoid cells. These observations confirm and extend previous reports of immunoglobulin expression in transfected cells (Cattaneo and Neuberger, 1987; De Waele *et al.*, 1988), and show that high-molecular-weight IgM-like complexes can be formed in the absence of the J chain. Previous studies of IgM expression by nonlymphoid transfectants have shown that absence of the J chain allows hexameric IgMs to accumulate, rather than the nearly exclusively pentameric chains found *in vivo* (Cattaneo and Neuberger, 1987). The presence of either IgG or IgM CH1 domains correlated with markedly reduced fusion expression, a result not entirely unexpected, given that CH1 is normally found associated with either a light-chain constant region or heavy-chain binding protein during the course of synthesis in B cells (Haas and Meo, 1988).

The purified fusion proteins potently inhibited virus infection and dissemination *in vitro*, and cultures treated with both fusion protein and complement fared better than cultures treated with either fusion protein alone or complement alone. In contrast, neither the IgG$_1$ nor the IgM fusion proteins showed significant complement-mediated cytotoxicity in a short-term chromium release assay. These results suggest that short-term assays may not measure relatively subtle or slow-acting effects that play an important role in long-term cultures, or that complement may participate in virus restriction by a mechanism divorced from cytolysis, *e.g.*, through interaction with specific receptors.

The inability to demonstrate a rapid direct cytolysis, despite high-affinity interaction and apparently normal binding of initial complement components (M. Leineweber and B. Seed, unpublished), is not unexpected. Even neutralizing antisera to HIV envelope determinants rarely show direct cytolytic activity in short-term *in vitro* assays (Nara *et al.*, 1987). Further, unlike most murine retroviruses, human retroviruses HIV-1 and HTLV-1 are little affected by exposure to human serum (Hoshino *et al.*, 1984; Banapour *et al.*, 1986). In a similar study (Capon *et al.*, 1989), it has been reported that a CD4:human IgG$_1$ CH1 fusion protein did not bind complement component C1q, and bound only to the high-affinity Fc receptors of the U937 cell line, which bears approximately twice as many low-affinity as high-affinity receptors (Vaughn *et al.*, 1985; Looney *et al.*, 1986; Fanger *et al.*, 1989). The reasons for these discrepancies are not known, but may be related to the presence of the CH1 domain, Traunecker *et al.* (1989), for example, found that a CD4:mouse IgG$_{2a}$ hinge fusion protein binds C1q. Further detailed analysis of the fusion proteins in HIV/SIV *in vitro* and *in vivo* models (see, for example, Watanabe *et al.*, 1989) will provide data about whether these molecules represent a realistic approach to AIDS therapy.

ACKNOWLEDGMENTS

We thank Sandro Aruffo for participation in the early phases of this project, and for useful suggestions; Rainer Jaenicke for communicating his results on analytical ultracentrifugation; Ernst Weinmann for assistance in the pharmacokinetic measurements; Gabriele Bergmann, Ludwina Preiss, Elke Veit, Ramona Volk, and Heike Wege for technical assistance; and Hans Küpper and Joachim Hilfenhaus for helpful discussions.

REFERENCES

ALLEN, J.M., and SEED, B. (1989). Isolation and expression of functional high-affinity Fc receptor complementary DNAs. Science **243**, 378–381.

BANAPOUR, B., SERNATINGER, J., and LEVY, J.A. (1986). The AIDS-associated retrovirus is not sensitive to lysis or inactivation by human serum. Virology **152**, 268–271.

CAPON, D.J., CHAMOW, S.M., MORDENTI, J., MARSTERS, S.A., GREGORY, T., MITSUYA, H., BYRN, R., LUCAS, C., WURM, F.M., GROOPMAN, J.E., BRODER, S., and SMITH, D.H. (1989). Designing CD4 immunoadhesins for AIDS therapy. Nature **337**, 525–531.

CATTANEO, A., and NEUBERGER, M.S. (1987). Polymeric immunoglobulin M is secreted by transfectants of non-lymphoid cells in the absence of immunoglobulin J chain. EMBO J. **6**, 2753–2758.

CHAKRABARTI, S., ROBERT-GUROFF, M., WONG-STAAL, F., GALLO, R.C., and MOSS, B. (1986). Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. Nature **320**, 535–537.

CHAUDHARY, V.K., MIZUKAMI, T., FUERST, T.R., FITZGERALD, D.J., MOSS, B., PASTAN, I., asnd BERGER, E.A. (1988). Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein. Nature **335**, 369–372.

CLAPHAM, P.R., WEBER, J.N., WHITBY, D., MCINTOSH, K., DALGLEISH, A.G., MADDON, P.J., DEEN, K.C., SWEET, R.W., and WEISS, R.A. (1989). Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T-cells and monocytes but not for brain and muscle cells. Nature **337**, 369–373.

CURRAN, J.W., JAFFE, H.W., HARDY, A.M., MORGAN, W.M., SELIK, R.M., and DONDERO, T.J. (1988). Epidemiology of HIV infection and AIDS in the United States. Science **239**, 610–616.

DEWAELE, P., FEYS, V., VAN DE VOORDE, A., MOLEMANS, F., and FIERS, W. (1988). Expression in non-lymphoid cells of mouse recombinant immunoglobulin directed against the tumor marker of human placental alkaline phoshatase. Eur. J. Biochem. **176**, 287–295.

DEEN, K.C., MCDOUGAL, J.S., INACKER, R., FOLENA-WASSERMAN, G., ARTHOS, J., ROSENBERG, J., MADDON, P.J., AXEL, R., and SWEET, R.W. (1988). A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature **331**, 82–84.

FANGER, M.W., SHEN, L., GRAZIANO, R.F., and GUYRE, P.M. (1989). Cytotoxicity mediated by human Fc receptors for IgG. Immunol. Today **10**, 92–99.

FISHER, R.A., BERTONIS, J.M., MEIER, W., JOHNSON, V.A., COSTOPOULOS, D.S., LIU, T., TIZARD, R., WALKER, B.D., HIRSCH, M.S., SCHOOLEY, R.T., and FLAVELL, R.A. (1988). HIV infection is blocked by recombinant soluble CD4. Nature **331**, 76–78.

GREGERSEN, J.P., WEGE, H., PREISS, L., and JENTSCH, K.D. (1988). Detection of human immunodeficiency virus and other retroviruses in cell culture supernatants by a reverse transcriptase microassay. J. Virol. Methods **19**, 161–168.

GREGERSEN, J.P., MEHDI, S., GELDERBLOM, H., and ZETTLMEISSL, G. (1990). A CD4:Immunoglobulin fusion protein with antiviral effects against HIV. Arch. Virol. **111**, 29–43.

HAAS, I.G., and MEO, T. (1988). cDNA cloning of the immunoglobulin heavy chain binding protein. Proc. Natl. Acad. Sci. USA **85**, 2250–2254.

HOSHINO, H., TANAKA, H., MIWA, M., and OKADA, H. (1984). Human T-cell leukemia virus is not lysed by human serum. Nature **310**, 324–325.

HUSSEY, R.E., RICHARDSON, N.E., KOWALSKI, M., BROWN, N.R., CHANG, H.-C., SICILIANO, R.F., DORFMAN, T., WALKER, B., SODROSKI, J., and REINHERZ, E.L. (1988). A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature **331**, 78–81.

LOONEY, R.J., ABRAHAM, G.N., and ANDERSON, C.L. (1986). Human monocytes and U937 cells bear two destinct Fc receptors for IgG. J. Immunol. **136**, 1641–1647.

NARA, P.L., ROBEY, W.G., GONDA, M.A., CARTER, S.G., and FISCHINGER P.J. (1987). Absence of cytotoxic antibody to human immunodeficiency virus infected cells in humans and its induction in animals after infection or immunization with purified envelope glycoprotein 120. Proc. Natl. Acad. Sci. USA **84**, 3797–3801.

SATTENAU, Q.J., and WEISS, R.A. (988). The CD4 antigen: Physiological ligand and HIV receptor. Cell **52**, 631–633.

SEED, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature **329**, 840–842.

SEED, B., and ARUFFO, A. (1987). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA **84**, 3365–3369.

SMITH, D.H., BYRN, R.A., MARSTERS, S.A., GERGORY, T., GROOPMAN, J.E., and CAPON, D.J. (1987). Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science **238**, 1704–1707.

STENGELIN, S., STAMENKOVIC, I., and SEED, B. (1988). Isolation of cDNAs for two distinct human Fc receptors by ligand affinity cloning. EMBO J. **7**, 1053–1059.

TILL, M.A., GHETIE, V., GREGORY, T., PATZER, E.J., PORTER, J.P., UHR, J.W., CAPON, D.J., and VITETTA, E.S. (1988). HIV-infected cells are killed by rCD4-ricin A chain. Science **242**, 1166–1168.

TRAUNECKER, A., LÜKE, W., and KARJALAINEN, K. (1988). Soluble CD4 molecules neutralize human immunodeficiency virus type 1. Nature **331**, 84–86.

TRAUNECKER, A., SCHNEIDER, J., KIEFER, H., and KARJALAINEN, K. (1989). Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. Nature **339**, 68–70.

VAUGHN, M., TAYLOR, M., and MOHANAKUMAR, T. (1985). Characterization of human IgG Fc receptors. J. Immunol. **135**, 4059–4065.

WATANABE, M., REIMANN, K.A., DELONG, P.A., LIU, T., FISHER, R.A., and LETVIN, N.L. (1989). Effect of recombinant soluble CD4 in rhesus monkeys infected with simian immunodeficiency virus of macaques. Nature **337**, 267–270.

ZETTLMEISSL, G., WIRTH, M., HAUSER, H., and KÜPPER, H.A. (1988). Efficient expression system for human antithrombin III in baby hamster kidney cells. Behring Inst. Mitt. **82**, 26–34.

Address reprint requests to:
Dr. Gerd Zettlmeissl
Research Laboratories of Behringwerke AG
D-3550 Marburg
West Germany

Received for publication December 19, 1989, and in revised form April 3, 1990.